Combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and canstatin gene suppression therapy on breast tumor xenograft growth in mice

Wen-Bo Wang · Yu-Lin Zhou · De-Feng Heng · Chuan-Hui Miao · Ying-Lin Cao

Abstract   Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene therapy and canstatin gene therapy have been investigated extensively in human xenograft tumor models established in immunocompromised nude mice. However, combination antitumor activity of these two agents and the safety of such gene constructs driven by the human telomerase reverse transcriptase (hTERT) promoter in nude mice have not been well documented. We hypothesized that TRAIL and canstatin gene therapy driven by the hTERT promoter might overcome the problem of liver toxicity and still effectively induce apoptosis on tumor cells. In this study, we evaluated the antitumor effects of TRAIL in human breast cancer cell lines and the antiangiogenic effects of canstatin on ECV204 cells. We also analyzed the effects of combined gene therapy using both TRAIL and canstatin in a human breast cancer nude mouse model. Tumor growth, tumor inhibition rate of each group, and toxicity were evaluated after gene therapy. Our results demonstrate that treatment using the canstatin- or TRAIL-expressing vector alone significantly suppresses tumor growth, compared to PBS or a vector control. We also found that combining these two therapies had greater antitumor activity than either treatment alone in the mouse model. Moreover, induction of apoptosis was not detected in normal mouse tissues after intratumoral injection of vectors and liver toxicity did not occur with either treatment. Thus, the combination of TRAIL and canstatin appears to be a promising approach for the gene therapy of breast tumors.

Keywords   Canstatin · TRAIL · hTERT · Targeted gene expression · Apoptosis · Antitumor activity

Introduction

Breast cancer is the most common malignancy and the second leading cause of cancer-related deaths among women [1]. Conventional therapy such as surgery, radiation, and chemotherapy have been widely used to induce apoptosis in breast tumor cells [2], but have not improved survival rates or successfully stalled higher rates recidivism or metastasis [3, 4], while the anti-cancer treatment may frequently be discontinued because of intolerable toxicity and/or development of drug resistance. Recently, different combined strategies have been investigated in order to improve the effects of chemotherapy and radiotherapy. Two agents that have been extensively investigated are the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and canstatin. However, preclinical and clinical studies have revealed that the application of these two agents is hampered by either weak anticancer activity or systemic toxicity. Therefore, the development of strategies to maximize the therapeutic index through increasing the target effect and minimizing toxicity is essential if these agents are to be used to successfully treat cancer.
As potent inducers of apoptosis, the canstatin and TRAIL proteins are both well-characterized molecules that elicit apoptosis through one of the two distinct apoptotic pathways. In the case of TRAIL, which is a type II transmembrane protein whose extracellular region forms a soluble molecule upon cleavage [5, 6], apoptosis is induced only in a wide variety of tumorigenic or transformed cell lines, but not in normal cells [7, 8]. This occurs via interaction with the death receptors, DR4/TRAIL-R1 and DR5/TRAIL-R2 [9–11], which in turn activate caspase-8 through the Fas-associated death domain, leading to apoptosis. Although the death domain containing receptors for TRAIL are expressed in both normal and cancer cells [12], and some toxicity to normal human hepatocytes was reported recently [13, 14], TRAIL is not cytotoxic to normal cells. This is thought to be due to the presence of the antagonistic decoy receptors DcR1, DcR2, and osteoprotegerin [15, 16] that inhibit TRAIL signaling in normal cells, but are absent on most tumor cells [17]. In contrast, canstatin, which is a 24-kDa peptide derived from the C-terminal globular non-collagenous (NC1) domain of type IV collagen chain 2, has been shown to induce apoptosis in cultured endothelial cells in vitro and to inhibit angiogenesis in vivo [18, 19]. In addition to its suppressive effects on EC proliferation and tube formation, migration, and proliferation, canstatin can also induce apoptosis in vitro, as well as suppress tumor growth in vivo. A previous study suggested that the ED50 of human canstatin is approximately 3-fold less than that of the well-known angiogenic inhibitor, endostatin, for halting the growth of tumors; and canstatin may be more potent than endostatin in tumor dormancy therapy.

Recent studies have shown that direct transfer of the TRAIL gene into cancer cells can elicit apoptosis, bystander effects, and suppressed tumor growth both in vitro and in vivo, while exerting few toxic effects on normal cells. These effects are primarily initiated by membrane-bound TRAIL [20], which is more effective against breast cancer cells. Furthermore, TRAIL induces apoptosis in human umbilical vein endothelial cells (EC) isolated from human umbilical veins or human dermal microvessels, tissues that express death domain-containing TRAIL-R1 and R2 [21]. Canstatin has also been associated with downmodulation of the anti-apoptotic protein, FLIP, through inhibition of the phosphorylation of Akt, FAK, mTOR, and its effectors; it induces Fas ligand expression and promotes TRAIL-induced apoptosis through a caspase and death receptor related apoptotic pathway [22], thus one can see that canstatin and TRAIL have the potential to interact positively during therapy, providing a basis for combined gene therapy. We recently found that breast cancer cell lines that are resistant to chemotherapy or to recombinant TRAIL protein are susceptible to TRAIL gene therapy. However, it is unclear whether a combination of TRAIL and canstatin gene treatment will lead to enhanced antitumor activity in breast cancer cells.

For this study, we hypothesized that simultaneous activation of the antiangiogenesis and extrinsic apoptotic pathways would elicit a more robust apoptotic response than activation of a single mechanism, thereby providing an opportunity to kill malignant cells that are relatively refractory to apoptosis. To test this hypothesis, we investigated the effects of vectors expressing the TRAIL or the canstatin genes under control of the human telomerase reverse transcriptase (hTERT) promoter on MCF-7 human breast cancer cells. The hTERT promoter is useful for targeting the therapy to cancer cells and preventing systemic damage to normal cells. Our results suggest that combined TRAIL and canstatin gene therapy has synergistic or additive effect on breast cancer cell lines and suppressed tumor growth with minimal toxicity in vivo. This may indeed represent a method to treat other tumors using the TRAIL and canstatin combination gene therapy.

Materials and methods

Cell lines and cell culture

The human MCF-7 breast tumor line, HELF human embryonic lung fibroblasts, and ECV204 human umbilical vein endothelial cells were all cultured in RPMI 1640 medium (Gibco BRL, Germany) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO2/95% air incubator. The breast tumor cells (MCF-7) were telomerase-positive; whereas HELF cells were telomerase-negative.

Construction of recombinant expression vectors

To construct the canstatin or TRAIL expression vector under control of the hTERT promoter, a luciferase expression vector (pHtERT-luc) driven by the hTERT was constructed by inserting hTERT promoter into a pGL3-Enhancer expression vector. The pEFGP-TRAIL expression vector containing the GFP:full length human TRAIL fusion, as reported previously [23], was used as a template. The 844 bp fragment of TRAIL was generated by PCR from pEFGP-TRAIL using a forward primer (5'-TACAAGCTTTATGGCCATGATGGAGGTCC-3') and a reverse primer (5'-TCTCTAAGGCCAACCTAAAAAGGCCCCG-3'); the resulting cDNA was digested with HindIII and NheI and...
ligated into pre-digested phTERT-luc instead of luciferase and designated as the phTERT-TRAIL expression vector. PSV40-TRAIL, which has an SV40 promoter and SV40 enhancer was constructed by replacing the luciferase gene of the pGL3-control plasmid with TRAIL. The expression vector, pETC, was used as described previously [24] and as a template. The 684 bp fragment of canstatin with a human IgG γ chain signal sequence was generated by PCR from pETC using forward (5'-GGCTAGCATGGAAGGCCCAAG CTCAAGCTTCTCTCCTCCTGCTACTCTGGTCACCAG ATACCAC CGGAGTCAGCATCGGC-3') and reverse primers (5'-CGCAAGCTTTCACAGGTTCTTCAT-3'); the resulting product was digested with HindIII and NheI, ligated into pre-digested phTERT-luc, and designated as the phTERT-canstatin secretory eukaryotic expression vector. Automated DNA sequencing was used to analyze DNA sequences at their junctions and to confirm the authenticity of each construct sequence.

Luciferase assay

The transcriptional activity of the hTERT promoter in breast cancer and fibroblasts cells was determined using luciferase reporter plasmids as described previously [25, 26]. Briefly, MCF-7 and HELF cells were plated at a density of 1 × 10^5 cells/ml 1 day prior to transfection with 0.5 μg of the luciferase reporter plasmids (pGL3 Luciferase Reporter Vectors, Promega ) co-transfected with 0.5 μg of pRL-TK control vector (pRL Family of Renilla Luciferase Control Reporter Vectors, Promega) plasmid. The following plasmids were used: the SV40 enhancer/promoter (pGL3-control) as a positive control, the hTERT promoter plasmids (phTERT-luc), and the negative control without promoter (pGL3-basic), as described previously [25]. The cells were harvested 48 h after transfection and washed twice with PBS, then lysed in 100 μl luciferase assay reagent II(LAR II) provided with the Dual-Luciferase Reporter Assay System (Promega). Transcriptional activity was measured using a Microtiter Plate Luminometer (Biochemistry Laboratories). Firefly luciferase protein catalyzes luciferin oxidation (M1). Then, 100 μl Stop and Glo Reagent was added to the cells, followed by measurement and sea pansy luciferase from pRL-TK, which catalyzes coelenterate luciferin (coelenterazine) oxidation to produce light (M2). The luciferase activity of the pGL3-control plasmid in each cell line was considered as 100%. The luciferase transcriptional activity was defined as the ratio of M1/M2 for each sample. All experiments were performed at least 3 times in each plasmid and represent the relative luciferase activity as an average.

RT-PCR analysis

The expression of TRAIL or canstatin mRNA was analyzed by semi-quantitative RT-PCR amplification. MCF-7 cells of the same quality were seeded into a 24-well plates and grown to 90–95% confluence, at which point the gene constructs (phTERT-TRAIL, phTERT-canstatin, pSV40-TRAIL, and phTERT-luc) were transfected by lipofection (Lipofectamine™ 2000, Invitrogen) according to standard procedures. After 48 h, the total RNA of transfected and untreated MCF-7 cells were extracted using TRIZOL reagent (GIBCO). RT-PCR for canstatin and TRAIL message was carried out using an AMV reverse transcription kit (Promega). The primers and cycle conditions were identical to those described above. The human β-actin gene, used as an internal control, was amplified by use of the primers: sense, 5'-GGCATCGTAGAGTGATCCG-3' and antisense, 5'-GCTGGAAGGTGGACAGCGA-3'. The amplified products were fractionated on a 2% agarose gel containing 0.5 mg/ml ethidium bromide. Gels were photographed and quantitatively scanned using imaging software for the analysis of canstatin and TRAIL gene expression in each group.

Western blotting

The protein expression of TRAIL and canstatin was analyzed by western blotting. phTERT-TRAIL, phTERT-canstatin, and phTERT-luc transiently expressing MCF-7 cells (1 × 10^6 cells/ml) and control MCF-7 cells were washed with cold PBS, harvested, counted, and lysed in 2x loading buffer, then incubated for 5 min at 99°C. Equal amounts of lysate were applied and run on an 10% sodium dodecyl sulfate polyacrylamide gel transferred to a nitrocellulose membrane (BioRad), and blocked with blocking buffer containing 5% low-fat milk and PBS with 0.05% Tween-20 for at least 1 h or overnight at 4°C, washed 3 times, then again with PBS containing 0.05% Tween-20, and incubated with TRAIL or canstatin antibodies (diluted 1:200) for at least 1 h at room temperature. After being washed again with PBST, the membranes were incubated with peroxidase-conjugated secondary antibodies and developed by DAB detection. β-actin was used as the loading control.

Endothelial cell proliferation assay

The procedures were performed as described previously [27]. Briefly, ECV204 cells were incubated in 96-well dishes at a density of 3.2 × 10^4 cells/well and incubated at 37°C with 5% CO2 and 95% humidity for 24 h. When the
incubated at 37°C. When the culture medium was replaced with fresh RPMI1640 and supernatant from the phTERT-canstatin and phTERT-luc transfection diluted in 100 μl RPMI1640 was added to each well. Control wells received only an equal volume of RPMI1640. After 36–48 h incubation, 20 μl MTT solution (5 mg/ml, Sigma) was added to each well and incubated for another 4 h. After the reaction, 100 μl medium was removed from each well and replaced with 100 μl DMSO, then vortexed gently for 10 min. The number of surviving cells was quantified by colorimetric MTT assay. All MTT experiments were performed in quadruplicate and repeated at least 3 times. Results of representative experiments are given as the mean ± SE.

Cell apoptosis assay

Fluorescence-activated cell sorting (FACS) was performed to determine the level of in vitro TRAIL expression and the number of apoptotic cells [28]. Briefly, MCF-7 and ECV204 cells were plated at densities of 2 × 10^5 and 3.2 × 10^4 cells/well plate one day prior to infection and incubated at 37°C with 5% CO2 and 95% humidity incubator for 24 h. The MCF-7 cells were then transfected with the recombinant expression vectors, phTERT-TRAIL, pSV40-TRAIL. ECV204 cells were then treated with the supernatant of phTERT-canstatin transfected cells. Both adherent and floating cells were harvested by trypsinization 48 h after treatment, and then MCF-7 cells were separated into two groups. One group was analyzed for TRAIL expression, while the other MCF-7s and ECV204 cells were gently washed twice with PBS and then fixed in 70% ethanol for 30 min. Cells were collected by centrifugation. RNase was added at 5 μg/ml and allowed to incubate for 1 h at 37°C, followed by staining with propidium iodide at 5 μg/ml. Staining was assessed by a FACStar plus flow cytometer (Becton Dickinson) and the results were analyzed with CellQuest software.

Animal experiments

Human breast cancer xenografts were established in 4–6-week-old nude mice (Experimental Animal Center of Academy of Sciences, Shanghai, China) by subcutaneous inoculation of 1 × 10^7 MCF-7 cells into the axilla of each mouse. After the tumor diameters reached 3–5 mm, the mice were divided randomly into five groups [PBS, phTERT-luc, phTERT-TRAIL alone, phTERT-canstatin alone, and canstatin plus TRAIL]. There were five mice in each treatment group. Treatment with the vector was performed by mixing the plasmid DNA (20 μg) with PBS in a volume of 100 μl for each mouse and injecting intratumorally 3 times every 3 days. Unless otherwise described, all of the experiments included PBS as a mock control and phTERT-luc as a vector control. When two vectors were used, the total vector amount remained the same, but individual vectors were mixed at a ration of 1:1. Tumors were measured with calipers, and tumor volumes were calculated (tumor volume = length × width^2 × 0.52) [29, 30]. The mice were sacrificed when tumors reached 2.0 cm in diameter or became ulcerated. The tumors were resected, and the inhibition rate of each group at the experiment end point was calculated.

Immunohistochemistry

Tumor specimens were fixed and frozen in Tissue Freezing Medium. About 5-μm cryosections were cut and stained with hematoxylin and eosin for histopathological analysis. In addition, assessment of histopathological changes in the heart, liver, spleen, lung, and kidney, as well as serum liver enzyme ALT and AST assays, were performed as previously described [31].

To analyze microvessel formation in tumors, sections were stained with anti-CD31 monoclonal antibody (Wuhan Boshide Biotechnology Co., Ltd) and subsequently with the ABC method. Positively-stained vascular endothelial cells (brown) were visualized and imaged using a digital camera attached to the microscope. The microvessel density was determined according to methods described elsewhere [32]. Briefly, regions of highest vessel density (“hotspot” regions) were scanned at low magnification (40 to 100×) and then counted at higher magnification (200×). Three such “hotspot” fields were counted in each tumor section, and the mean microvessel density value was recorded. Any endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel. Positively-stained vascular endothelial cells were visualized and imaged using a Magnifire camera attached to the microscope.

TUNEL staining

Tumor samples were cryopreserved in liquid nitrogen and cut into 8 μm thick slices. Slices were incubated in ice-cold 0.1% Triton X-100 and 0.1% sodium citrate for 8 min, then the slides twice with PBS, the area around the sample was dried, and 50 μl TUNEL reaction buffer containing enzyme and label solution was used according to the manufacturer’s protocol (Boehringer Mannheim) in a
humid box for 1 h at 37°C. Slides were observed by fluorescence microscopy after complete washing. The apoptotic index (AI) was calculated as follows: 
\[ \text{AI} = \frac{\text{number of apoptotic cells}}{\text{total number}} \times 100\% . \]

Statistical analysis

For in vivo experiments, tumor volumes were expressed as the mean ± SE. The Student’s t-test was used to test statistical significance of the differences between groups (two-tailed). The level of significance was set at \( P < 0.05 \).

Results

Transcriptional activity of the hTERT promoter in hTERT-positive or -negative cells

To confirm that the transcriptional activity of hTERT is observed only in hTERT-positive breast cancer cells, a transient transfection of the luciferase reporter plasmids was performed. The transcriptional activity of the SV40 enhancer/promoter positive control in each cell line was considered as 100%. As shown in Fig. 1, the hTERT promoter construct displayed robust transcriptional activity in the hTERT-positive MCF-7 tumor cell line, with a relative luciferase activity 112.92% of the positive control (pGL3-control); this difference was not statistically significant. In contrast to the transcriptional activity of hTERT-positive cells, the activity observed in HELF hTERT-negative cells was very low (4.45%). These results confirm that activation of hTERT transcription is significantly up-regulated only in hTERT-positive cells. SV40 and hTERT promoter activity differed by only 2- to 10-fold in cancer cells compared with a more than a 500-fold difference in normal cells. Together, these results demonstrate that the hTERT promoter is highly active in a variety of cancer cell lines, but not in normal cells, suggesting that the hTERT promoter is indeed both strong and specific enough for targeting transgene expression to tumors (Fig. 1).

hTERT promoter-driven TRAIL gene expression and induction of apoptosis in cancer and HELF cells in vitro

To determine the level of transgene expression and apoptosis induction in cancer and normal cells, cells were harvested 48 h after treatment with phTERT-TRAIL and the expression of TRAIL was analyzed by RT-PCR, western blot analysis, and FACS. Also, pSV40-TRAIL was used as a positive control for apoptosis induction because we have previously found that the SV40 promoter has transcription activity in most normal cells. PBS alone or phTERT-luc were used as mock and vector controls. Overexpression of the TRAIL protein was observed only in MCF-7 cells treated using phTERT-TRAIL or pSV40-TRAIL. As anticipated, there was obvious expression of TRAIL in HELF cells treated with pSV40-TRAIL (Fig. 2a). FACS analysis confirmed that less than 2% of the cells were TRAIL-positive after treatment using phTERT-TRAIL in HELF cells; however, more than 10% of the HELF cells treated with pSV40-TRAIL were positive. In comparison, more than 18% of the MCF-7 cells were TRAIL-positive after treatment with phTERT-TRAIL. These results are consistent with our previous observations that the hTERT promoter is highly active in cancer cells, but relatively quiescent in normal cells in vitro (Fig. 2b). FACS analysis demonstrated that treatment with phTERT-TRAIL, phTERT-luc, pSV40-TRAIL, and PBS all resulted in no more than a background level of cell killing in normal HELF cells (Fig. 2b). In comparison, substantial apoptosis was induced when MCF-7 cells were treated using phTERT-TRAIL. The incidence of apoptosis in these cells driven by the hTERT promoter system was as strong as the SV40 promoter in MCF-7 cells. The results of this assay were consistent with analyses based on observation of morphological changes (Fig. 2c), including condensation of chromatin at the margins of the nuclei, chromatin crescent formation, fragmentation of the nucleus, and cytoplasmic vacuoles that were observed in MCF-7 cells transfected with phTERT-TRAIL. However, there were no obvious changes in cells transfected with phTERT-luc or PBS, which displayed and had normal cell structures. Thus, the hTERT promoter can be used to drive tumor-specific
proapoptotic gene expression to induce apoptosis, while preventing off-target transgene expression and related toxicity in normal cells.

hTERT promoter-driven canstatin gene expression and apoptosis induction in endothelial cells in vitro

Treatment with phTERT-canstatic induced obvious expression of canstatin mRNA (RT-PCR) and protein (western blot) in MCF-7 cells (Fig. 3a). Compared to conditioned medium from MCF-7 cells transfected with phTERT-luc control, the conditioned medium from phTERT-canstatic MCF-7 cells was able to significantly suppress the S phase fraction (20 ± 2.4 vs. 6 ± 1.5%, \( P < 0.05 \)) in ECV204 cells (Fig. 3b). In contrast, when MCF-7 cells were treated with either of the two conditioned mediums, there were no differences in apoptosis or cell cycle distribution, suggesting that canstatin treatment
has no effect on MCF-7 tumor cell apoptosis or cell cycle
distribution. After 24 h incubation, canstatin conditioned
media inhibited ECV204 cell proliferation by 45.6 ± 9.2%,
while conditioned media derived from cultures of MCF-7
cells transfected with the phTERT-luc control vector had
no effect on endothelial cell proliferation; the rate of
inhibition was 8.5 ± 0.5% ($P < 0.05$, Fig. 3c). It thus
appears that canstatin can effectively inhibit the prolifera-
tion of endothelial cells.

TRAIL gene therapy combined with canstatin inhibited
tumor growth in vivo

We next tested the in vivo therapeutic efficacy of combi-
nation treatment with both phTERT-TRAIL and phTERT-
canstatin in a human MCF-7 xenograft mouse model. As
shown in Fig. 4, tumors treated with phTERT-TRAIL
combined with phTERT-canstatin grew very slowly in
nude mice; the inhibition rate on tumor size was 82% com-
pared to the PBS group at the end of the experiment
(Fig. 4a). The tumor volume of the combined phTERT-
TRAIL with phTERT-canstatin group (235 ± 59 mm$^3$),
was significantly different from that of the PBS group
(1420 ± 210 mm$^3$, $P < 0.01$) and the phTERT-luc group
(1100 ± 260 mm$^3$, $P < 0.01$). Tumors of the phTERT-
TRAIL only and phTERT-canstatin only groups also grew
more slowly than those of the PBS group or the phTERT-
luc group, but the effect on tumor growth was slightly
weaker than with the combined therapy group (66% and
62%, respectively). However, tumor volumes of the phT-
ERT-TRAIL group (485 ± 91 mm$^3$) or the phTERT-
canstatin group (520 ± 40 mm$^3$) was significantly different
from the PBS group ($P < 0.05$) and the phTERT-luc group
($P < 0.05$, Fig. 4b). These results suggested that syner-
gistic antitumor activity was elicited by the combined
treatment, corresponding to the inhibition of endothelial
cell proliferation and apoptosis-inducing activity.

Enhanced apoptosis induction and antiangiogenic
effects of combination TRAIL therapy and canstatin
gene

To test whether the enhanced cell killing produced by
combination treatment was due to apoptosis, we quantified
the sub-G1 population of MCF-7 cells after treatment with
TRAIL and canstatin gene therapy by flow cytometry. As

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**Fig. 3** Transgene expression and induction of apoptosis in MCF-7
and ECV204 cells. (a) Expression of canstatin in the MCF-7 cell line.
The expression of canstatin mRNA and proteins were assessed by RT-
PCR analysis (upper panel) and Western blots (low panel). Lane 1,
PBS; lane 2, phTERT-luc; lane 3, phTERT-canstatin. β-actin was
used as a positive control (lanes 4–6). (b) Canstatin induces
endothelial cell apoptosis in vitro. ECV204 cells were treated using
various vectors and tested for apoptosis by analyzing the cellular
DNA content by FACS. Top, treatments; number within each panel,
percentage of apoptotic cells. This experiment was repeated three
times and representative data are shown. (c) Inhibitory effects of
canstatin on the proliferation of endothelial cells. 1: Proliferation was
inhibited by conditioned media derived from cultures of MCF-7 cells
transfected with phTERT-luc; 2: proliferation was inhibited by
conditioned medium derived from cultures of MCF-7 cells transfected
with phTERT-canstatin; 3: control group. Values represent the means
of three determinations ($n = 3$) by MTT assay.
shown in Fig. 5, phTERT-TRAIL or phTERT-canstatin alone induced a <20% sub-G1 accumulation. However, combined treatment with phTERT-TRAIL and phTERT-canstatin resulted in a substantially increased sub-G1 accumulation (26%).

To determine whether the antitumor effect of canstatin and TRAIL combined therapy was associated with suppression of angiogenesis, the status of vessel formation in treated MCF-7 xenografts was assessed by immunohistochemistry using the endothelial cell-specific CD31 (PECAM) marker for staining. A representative immunostaining micrograph is shown in Fig. 6. We observed a high density of microvessels in the PBS and control vector groups; whereas canstatin or TRAIL treatment alone resulted in a significant reduction in vascularization (Fig. 6a–d). When canstatin and TRAIL were used together, complete suppression of tumor angiogenesis was achieved (Fig. 6e). The microvessels density in the phTERT-canstatin + phTERT-TRAIL combined and phTERT-canstatin alone group were significantly lower than in other groups (Fig. 6f).

The histology of this tumor during passage in nude mice mirrored that seen in the original specimen. Although smaller than the control tumors, tumors treated with

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**Fig. 4** Canstatin and TRAIL suppress tumor growth in an MCF-7 xenograft mice model. (a) Subcutaneous tumors derived from MCF-7 cells were treated with various reagents as indicated. The intratumoral injection of phTERT-canstatin, phTERT-TRAIL or phTERT-canstatin plus phTERT-TRAIL inhibited the growth of MCF-7 breast cancer compared with the PBS and phTERT-luc control. (b) Tumor volumes were monitored over time after the treatments. The values shown represent the means ± the standard error of five mice per group. The mean tumor volume in the animals treated with phTERT-TRAIL or phTERT-canstatin alone differed significantly from that of the phTERT-luc treated group (*P < 0.05). Treatment with phTERT-TRAIL plus phTERT-canstatin differed significantly from all other treatment groups (*P < 0.05).

**Fig. 5** Combination of TRAIL and canstatin-enhanced apoptosis induction in vivo. The effect of canstatin and TRAIL on the apoptosis of transplanted tumors in vivo. Tumors were treated using various vectors. (A) phTERT-canstatin plus phTERT-TRAIL; (B) phTERT-TRAIL; (C) phTERT-canstatin; (D) PBS; (E) phTERT-luc; number within each panel, percentage of apoptotic cells. The typical apoptotic peak was observed in groups A, B and C. The apoptotic rate in the canstatin combined with TRAIL (group A) was higher than that in groups B, C, D and E (*P < 0.05).
TRAIL or canstatin alone consisted mainly of viable tumor cells and relatively little stroma. In contrast, tumors treated with TRAIL and canstatin were significantly smaller and consisted of mainly stromal elements with only a few scattered tumor necrosis cells. We observed large numbers of apoptotic cells that were mainly in the center of the tumor with nuclear condensation and eosinophilic cytoplasm. However, growing tumor cells flourished in the PBS or phTERT-luc alone groups. Thus, apoptosis was indeed responsible for the cell death produced by the combination of TRAIL gene therapy and canstatin, and this also suggested that the combination treatment enhanced apoptosis.

Combination of TRAIL and canstatin-enhanced apoptosis induction in vivo

To assess the induction of apoptosis in vivo, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining on tumor sections from all five treatment groups (Fig. 7a). The strong fluorescent green color indicates apoptotic nuclei, as visualized using 3,3’-diaminobenzidine substrate. Apoptotic cells were counted under a fluorescence microscope in randomly chosen fields; the apoptotic index was calculated as the percentage of at least 1,000 scored cells. As shown in Fig. 7b, the combination of TRAIL and canstatin resulted in a significantly higher apoptotic index (5.17%) compared with TRAIL (2.99%) or canstatin (2.85%) alone, phTERT-luc (1.85%), or PBS (1.67%) after subtracting the background level of 2.2% (∗P < 0.05).

We also detected the protein expression of TRAIL and canstatin from the hTERT promoter after intratumoral administration by immunohistochemical analysis of tumor specimens from the same five treatment groups. We observed robust expression of TRAIL protein in tumor tissues obtained from animals that were treated with phTERT-TRAIL combined with phTERT-canstatin or treated with phTERT-TRAIL alone (Fig. 7c). Similarly, high-level expression of canstatin protein was found in tumors treated with phTERT-canstatin alone or combined with TRAIL. As expected, we did not observe TRAIL expression in tumors that were treated with PBS or phTERT-luc.

Toxicity and transgene expression after intratumoral vector administration

We also evaluated the possible toxicity of these treatments by measuring the serum content of the liver enzymes...
alanine transaminase (ALT) and aspartate transaminase (AST). For this testing, serum samples were collected after the treatment schedule ended. Analysis of the serum ALT and AST level showed that they were both within the normal range for all of the treatment groups (Fig. 8a), suggesting that the treatment was well tolerated. We also examined histopathological changes in the heart, liver, spleen, lung, and kidney after these organs were collected (Fig. 8b). We noticed no other pathologic changes and found no substantial differences in any of the organs in any treatment group, suggesting that the treatments were well tolerated. Taken together, these results suggest that intratumoral administration of the TRAIL and canstatin genes separately or combined results in minimal toxicity when transgene expression is controlled by the hTERT promoter. Furthermore, we were able to detect TRAIL or canstatin transgene expression only in treated tumors, but not in normal treated tissue or tumors treated with only the
control vector or PBS. These results confirm the specificity of the targeted gene expression achieved by using the hTERT promoter in intratumoral vector administration, corresponding to our results in vitro.

Discussion

A major mode of resistance to antitumor therapy is insensitivity to apoptosis induction [33]. Apoptosis, a genetically regulated mechanism of cell death, is initiated through two major pathways: the intrinsic mitochondrial pathway and extrinsic membrane pathway [34, 35]. A variety of genes are involved in the conduction of these two pathways, some with proapoptotic functions and others with antiapoptotic function. The relative balance of these competing activities determines the cell fate, to die or not to die [36]. Therefore, enforced, selective overexpression of proapoptotic genes involved in these pathways can effectively change this balance, leading to apoptosis of transfected malignant cells. However, tumor growth and metastasis also depend largely upon blood supply and vessel formation [37, 38]. Achieving highly efficient angiostatic and antitumor effects with currently available antiangiogenic factors remains challenging. Thus, angiogenesis inhibitors have emerged as a potent new class of therapeutics that thwart tumor growth by blocking the formation and growth of new blood vessels, made attractive by the direct endothelial targeting and absence of drug resistance [39, 40]. One possible new strategy in the treatment of breast cancer is to combine angiogenesis inhibitors and proapoptotic genes that may trigger apoptosis at different levels or through different pathways, thereby enhancing induction of apoptosis and perhaps improving the therapeutic outcome.

Recently, some research efforts have now been focused on combined multi-modality treatment in an attempt to deal with challenge posed by breast cancer. For example, gene therapy, used as either primary therapy or adjuvant therapy in combination with conventional therapy, may prove beneficial. The attraction of gene therapy is that by inducing local intra-tumoral expression of the desired therapeutic proteins, a constant therapeutic effect may be produced at the cancer site, while reducing systemic toxicity. This approach would greatly increase the number of proteins that may be used in cancer treatment, as it would allow use of many proteins that would otherwise not be tolerated by the patient.

We recently found that both doxorubicin-sensitive and doxorubicin-resistant breast cancer cell lines that are resistant to recombinant TRAIL proteins are susceptible to TRAIL gene therapy expressed from the hTERT promoter [41], suggesting that TRAIL is a potent antitumor agent for both chemosensitive and chemoresistant tumors. Treatment of breast cancers using the TRAIL gene was recently reported by others [42]. Importantly, the results of these studies made clear that the antitumor activity of the TRAIL gene could be further enhanced when combined with chemotherapy [43] or radiation [44, 45], acting on endothelial cells [46]. Furthermore, transgene expression and apoptosis induction by TRAIL was minimal in normal human fibroblasts, normal human primary hepatocytes, and normal mammary epithelial cells in culture. In the present study, we evaluated the therapeutic and toxic effects of the TRAIL and canstatin genes both separately and combined in the treatment of breast tumors derived from MCF-7 breast cancer cell xenografts.

Similar inhibitory effects of tumor growth have been obtained with angiogenesis inhibitors [47]. Canstatin, one such factor, was recently shown to inhibit endothelial cell proliferation, migration, and tube formation, with no inhibition of proliferation or apoptosis on non-endothelial cells in vitro and to suppress the primary and metastatic growth and vascularization of human tumor xenografts in vivo [48]. In this respect, the studies showed that the density of vascularity decreased and the transplanted tumors were highly suppressed by antiangiogenic therapy combined with chemotherapy or interleukins. However, at present, most researchers continue to make recombinant canstatin in prokaryotic expression systems. Because of this limitation, one must repeatedly inject high-dose recombinant protein for clinical application. This is rather difficult and, in a way, limits the application of canstatin protein. However, gene therapy can overcome these limitations by local intratumoral injection therapy. The results of the present study suggest that the antiangiogenic gene, canstatin, significantly inhibits the growth of established MCF-7 tumors, and that the antitumor effects of canstatin can synergize with TRAIL gene therapy, leading to substantial suppression of xenograft growth.

We used a luciferase expression vector (pHTERT-luc) driven by an hTERT promoter that was constructed from the pGL3-Enhancer, which is known to increase hTERT promoter activation [49]. Furthermore, our results demonstrate for the first time that treatment using either the TRAIL or canstatin gene alone driven by the hTERT promoter was able to significantly suppress tumor growth. Moreover, combined TRAIL and canstatin therapy further significantly suppressed tumor growth when compared with either TRAIL or canstatin therapy alone. Neither of these therapies had any detectable toxicity. Altogether, any synergy that results from combined therapy is expected to improve the overall therapeutic results by reducing toxicity, enhancing efficacy, or both. Our present results support a model whereby canstatin combined with the TRAIL gene can be a useful approach in hTERT-positive tumor therapy.
This is not restricted to breast cancer and might be expected to function in other cancer therapies, providing a new strategy for cancer prevention and cure. Thus, with the progress made in gene therapy and other biomedical fields, treatment of breast cancers will, in the future, become more effective and realistic. We believe that combination TRAIL and canstatin gene therapy may have clinical applications.

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